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Tissue Transglutaminase, Coagulation Factor XIII, and the Pro-polypeptide of von Willebrand Factor Are All Ligands for the Integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$*

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We previously reported that MOLT-3 human lymphocyte-like leukemia cells adhere to tissue-type transglutaminase (tTG) through the integrin $\alpha_4\beta_1$. We now report that G-361 human melanoma cells also adhere to tTG, although they do not express $\alpha_4\beta_1$. G-361 cells utilize two additional integrins, $\alpha_9\beta_1$ and $\alpha_5\beta_1$ to adhere to tTG. Furthermore, blood coagulation factor XIII (FXIII), another member of the transglutaminase family that is highly homologous to tTG, and propolypeptide of von Willebrand factor (pp-vWF) also promoted cell adhesion through $\alpha_9\beta_1$ or $\alpha_4\beta_1$ in G-361 or MOLT-3 cells, respectively. In the case of pp-vWF, $\alpha_9\beta_1$ and $\alpha_4\beta_1$ both bind to the same site, comprised of 15 amino acid residues and designated T2–15. Moreover, SW480 human colon cancer cells stably transfected to express $\alpha_9\beta_1$, but not mock transfectants, adhered to tTG, FXIII, pp-vWF, and T2–15/bovine serum albumin conjugate. These data identify tTG, FXIII, and pp-vWF as shared ligands for the integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$. This report is the first to unambiguously show that these two integrins share the same cell adhesion site within one protein and provides strong support for classifying $\alpha_9\beta_1$- and α_4-integrins as functionally related members of an integrin subfamily.

Integrins are a family of heterodimeric transmembrane receptors that mediate cell-extracellular matrix and cell-cell interactions and play important roles in a wide variety of cellular events (1–6). Each integrin is composed of noncovalently associated α and β subunits, and the combination of α and β subunits generates many different receptors with different ligand specificity.

Integrin α subunits can be grouped into subfamilies based on sequence similarity, and these subfamilies generally define integrins that share common ligands. Thus, α subunits can be divided into five groups (6–8): the first group (α_1, α_2, α_{10}, and α_{11}) recognizes collagen, the second group (α_3, α_6, and α_7) recognizes laminin, the third group (α_5, α_8, α_v, and α_{IIb}) recognizes RGD-containing sequences, and the fourth group (α_L, α_M, α_X, and α_D) recognizes ICAM-1. α_4 and α_9 are the only members of the fifth group (9). Somewhat surprisingly, the initial ligands identified for α_9 and α_4-containing integrins did not appear to overlap. Thus, for example, the integrin $\alpha_4\beta_1$ was found to recognize fibronectin (10, 11) and the vascular cell adhesion molecule-1 (VCAM-1)[1] as ligands (12), whereas the integrin $\alpha_9\beta_1$ was reported to recognize tenascin-C, (13, 14), and osteopontin (15, 16). However, recently Bayless *et al.* (17) reported that $\alpha_4\beta_1$ recognizes osteopontin as a ligand, and Taooka *et al.* (18) reported that $\alpha_9\beta_1$ recognizes VCAM-1. It thus appears that the $\alpha_4\beta_1$- and $\alpha_9\beta_1$-integrins, like other integrins that are related based on α subunit sequence homology, do share at least some common ligands.

In the present study, we demonstrate that three additional proteins are ligands for both $\alpha_9\beta_1$ and $\alpha_4\beta_1$. The first is a tissue-type transglutaminase (tTG). This protein belongs to a family of transglutaminases (EC 2.3.2.13) that catalyze ϵ-(γ-glutamyl)lysine cross-link formation between specific substrate proteins (19–21) and are distributed widely in various tissues. The second is blood coagulation factor XIII (FXIII). This protein is also a member of the transglutaminase family and has an important role in the final stage of the blood coagulation cascade. The last is the propolypeptide of von Willebrand factor (pp-vWF). This protein is obtained from a large precursor of von Willebrand factor by specific cleavage during biosynthesis and is stored in granules of both endothelial cells and platelets (22, 23). In the case of pp-vWF, we have mapped the recognition sequence for both integrins to the same cell adhesion site, a 15-residue linear sequence that we have previously shown is required for $\alpha_4\beta_1$-mediated adhesion to this protein (24).

EXPERIMENTAL PROCEDURES

Materials—tTG was purified from guinea pig liver using an anti-tTG monoclonal antibody (mAb) 8D, which was a gift from Dr. K. Ikura (Kyoto Institute of Technology, Kyoto, Japan), raised against guinea pig liver tTG as described (25). Human placental FXIII was kindly provided from Hoechst Japan Co. (Tokyo, Japan) and was further purified as described (26). pp-vWF was purified from washed bovine platelets by immunoaffinity chromatography, as described (27). Porcine vitronectin was a gift from Dr. M. Hayashi (Ochanomizu University, Tokyo, Japan). Mouse laminin, RGD peptide (GRGDSP), and RGE peptide (GRGESP)

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[1] The abbreviations used are: VCAM-1, vascular cell adhesion molecule-1; tTG, tissue-type transglutaminase; FXIII, blood coagulation factor XIII; pp-vWF, propolypeptide of von Willebrand factor; mAb, monoclonal antibody; BSA, bovine serum albumin.

were purchased from Iwaki Glass (Tokyo, Japan).

Antibodies—Mouse mAb 4B4, which recognizes human β_1-integrin, was a gift from Dr. C. Morimoto (Dana Farber Cancer Institute, Boston, MA). The hybridoma cell line secreting mAb TS2/16, which recognizes and activates human β_1-integrin (28), was obtained from the American Type Culture Collection. This mAb was obtained in ascites and purified by ammonium sulfate precipitation and anion exchange chromatography. Mouse anti-human $\alpha 2$ subunit mAb 6F1 was a gift from Dr. B. S. Coller (Mount Sinai School of Medicine, New York, NY). Mouse anti-human α_3 subunit mAb, P1B5, mouse anti-human α_4 subunit mAb HP2/1, and mouse anti-human α_5 subunit mAb, P1D6, were purchased from Telios Pharmaceuticals, Inc. (La Jolla, CA), Cosmo Bio Co., LTD. (Tokyo, Japan), and Life Technologies, Inc. (Tokyo, Japan), respectively. Rat anti-human and -mouse α_6 subunit mAb, GoH3, was a gift from Dr. A. Sonnenberg (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Mouse anti-human α_9 subunit mAb, Y9A2, was generated and characterized as described previously (29). Polyclonal antibody against human vitronectin receptor ($\alpha_v\beta_3$) was purchased from Telios Pharmaceuticals Inc.

Cell Lines and Cell Culture—G-361 human melanoma cells and MOLT-3 human lymphoma cells were provided from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and cultured in RPMI 1640 medium containing 10% fetal calf serum and nonessential amino acids. α_9-transfected or mock transfected SW480 human colon cancer cells were previously described in Ref. 13. These cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1 mg/ml neomycin analog, G418 (Life Technologies, Inc.).

Peptide Synthesis—Peptides of CS-1 (EILDVPST), located in an alternatively spliced segment of fibronectin, and TNfnIII3 (AEIDGIELTYG) located in the third fibronectin type III repeat of tenascin-C, were manually synthesized by the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-based solid phase method. T2–15 peptide (DCQDHSFSIVIETVQ) was synthesized as described previously (24). Bovine serum albumin (BSA) conjugated with CS-1 peptide, TNfnIII3 peptide, or T2–15 peptide (CS-1/BSA, TNfnIII3/BSA, or T2–15/BSA, respectively) was prepared in our laboratory.

Cell Adhesion Assay—Cells were washed three times and suspended in serum-free RPMI 1640 or Dulbecco's modified Eagle's medium containing 0.25 mM $MnCl_2$ prior to the cell adhesion assay. In the case of MOLT-3 cells, in addition to 0.25 mM $MnCl_2$, 5 μg/ml TS2/16, a β_1-integrin activating mAb, was added to the cell suspension. Cell adhesion assays were performed according to the method described previously (30). In brief, 6-mm square chips cut from bacteriologic plastic dishes (Falcon 1029) were coated for 16 h at 4 °C with 50 μl of solution containing adhesive proteins at the following concentrations diluted in 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Laminin and vitronectin were 5 μg/ml; tTG and pp-vWF were 10 μg/ml; TNfnIII3/BSA, CS-1/BSA, and T2–15/BSA were 20 μg/ml; FXIII was 30 μg/ml. More than 80% of ligands were absorbed on the surface under these conditions. After blocking nonspecific protein binding sites by incubation with 1% BSA at room temperature for 1 h, chips were placed in 48-well tissue culture dishes (Costar 3548) and overlaid with 2–5 × 10^4 cells in 50 μl of serum-free medium. After incubation at 37 °C for 90 min, chips were picked up, rinsed in phosphate-buffered saline to remove nonadherent cells, and fixed with 1% glutaraldehyde in phosphate-buffered saline. Cells on chips were stained, when necessary, with 0.5% crystal violet in 20% methanol. Adherent cells were either photographed or counted using a light microscope with a calibrated grid marked on the ocular lens. When inhibitory antibodies were used in adhesion assays, cells were first incubated with antibody at 37 °C for 30 min. Concentrations of synthetic peptides and inhibitory antibodies used in this study, except Y9A2, were 1 mM and 10 μg/ml, respectively. Y9A2 was used as a 1:50 dilution of hybridoma supernatant.

Flow Cytometry—Flow cytometry was performed according to the method described previously (31). Cells were washed as described above and treated with an appropriate primary antibody in serum-free medium for 30 min on ice. After removal of unbound antibody, cells were incubated with 1:100 diluted fluorescein isothiocyanate-conjugated anti-mouse IgG for 30 min on ice, washed, and analyzed on a FACScan flow cytometer (Beckton Dickinson and Co.).

RESULTS

G-361 Human Melanoma Cells Adhere to tTG through β_1-Integrin(s) but Not $\alpha_4\beta_1$—In a previous study (31), we reported that tTG promoted cell adhesion of MOLT-3 cells mediated by the integrin $\alpha_4\beta_1$. To determine whether adhesion of all cells to

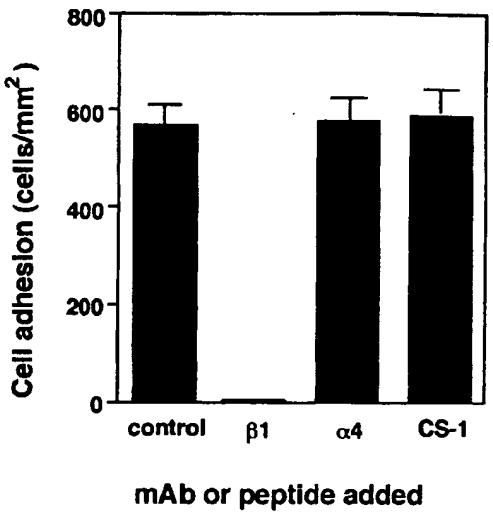

FIG. 1. **Adhesion of G-361 cells to tTG is not mediated by $\alpha_4\beta_1$-integrin.** Cell adhesion was determined in the presence of no mAb (*control*), an anti-β1 mAb 4B4 (*β1*), an anti-α4 mAb HP2/1 (*α4*), or CS-1 peptide (*CS-1*). The data represent the means ± S.E. of one of the representative experiments in which triplicate determinations were made.

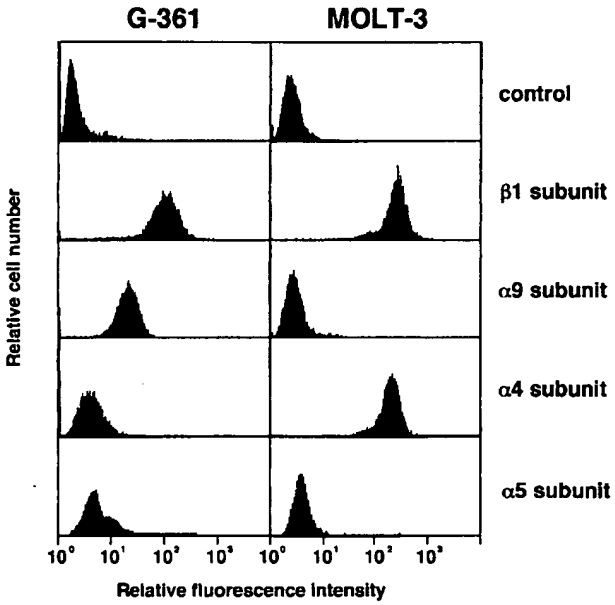

FIG. 2. **Flow cytometry of integrins expressed on G-361 cells and MOLT-3 cells.** Cells were reacted with no IgG (*control*), an anti-human β_1 mAb TS2/16 (*β1 subunit*), an anti-human α_9 mAb Y9A2 (*α9 subunit*), an anti-human α_4 mAb HP2/1 (*α4 subunit*), or an anti-human α_5 mAb P1D6 (*α5 subunit*).

tTG is dependent on $\alpha_4\beta_1$, we performed adhesion assays with a variety of cell lines in the presence and absence of anti-α_4 blocking antibody. Although adhesion of most cells was inhibited by an anti-α_4 mAb, there was one notable exception. Adhesion of G-361 human melanoma cells to tTG was not inhibited at all by this mAb (Fig. 1). Furthermore, this adhesion was not inhibited by CS-1 peptide, which inhibits $\alpha_4\beta_1$-mediated adhesion. However, G-361 cell adhesion to tTG was completely inhibited by anti-β_1 antibody. These results suggest that one or more β_1-containing integrin, other than $\alpha_4\beta_1$, is responsible for G-361-mediated adhesion to tTG.

Expression of $\alpha_9\beta_1$ and α_5 Subunit on G-361 Cells—The β_1-integrin family includes at least 12 heterodimers composed

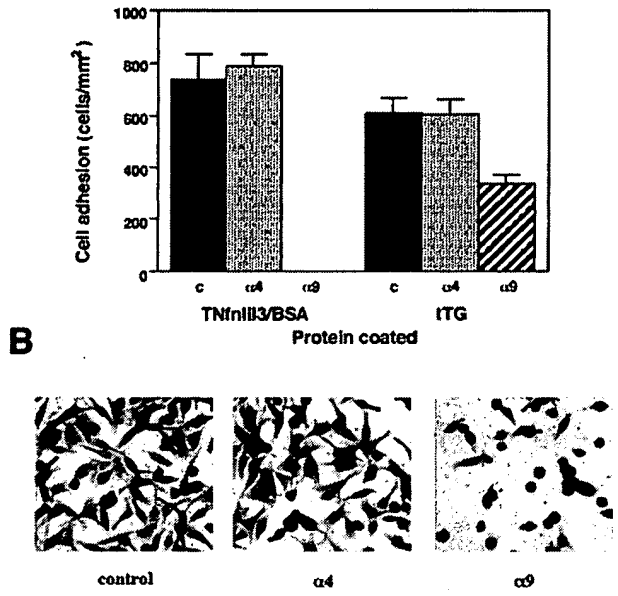

FIG. 3. **The effect of anti-α_9 subunit mAb on G-361 cell adhesion to tTG.** *A*, adhesion of G-361 cells to TNfnIII3/BSA or tTG was determined in the presence of no mAb (*black columns*, *c*), an anti-α4 mAb HP2/1 (*gray columns*, *α4*), or an anti-α9 mAb Y9A2 (*hatched columns*, *α9*). The data represent the means ± S.E. of one of the representative experiments in which triplicate determinations were made. *TNfnIII3/BSA*, TNfnIII3 peptide-BSA conjugate. *B*, photographs show morphology of G-361 cells adhering to tTG in the presence of mAbs.

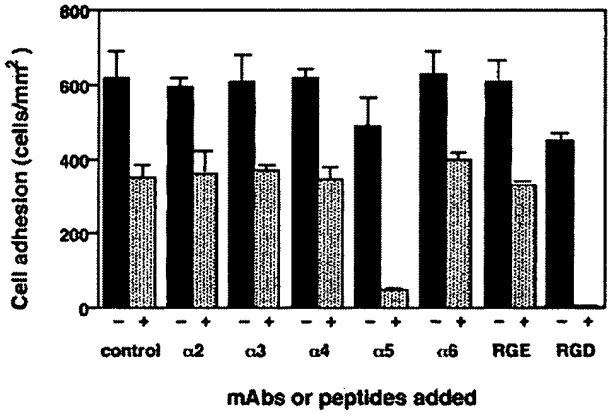

FIG. 4. **Identification of integrins involved in cell adhesion of G-361 to tTG.** The effect of mAbs and peptides on adhesion of G-361 cells to tTG was determined in the absence (*black columns*, −) or presence (*gray columns*, +) of anti-α_9 mAb Y9A2. The following mAbs or peptides were used: *control*, without mAb; *α2*, an anti-α_2 mAb 6F1; *α3*, an anti-α_3 mAb P1B5; *α4*, an anti-α_4 mAb HP2/1; *α5*, an anti-α_5 mAb P1D6; *α6*, an anti-α_6 mAb GoH3; *RGE*, RGE peptide (GRGESP); *RGD*, RGD peptide (GRGDSP). The data represent the means ± S.E. of one of the representative experiments in which triplicate determinations were made.

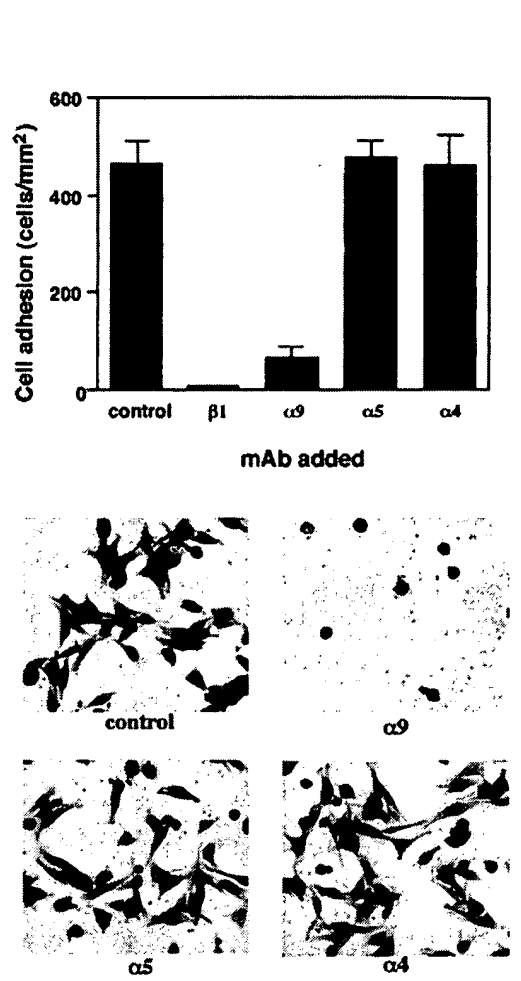

FIG. 5. **Identification of integrins involved in cell adhesion of G-361 to FXIII.** *A*, adhesion of G-361 cells to FXIII was determined in the presence of no mAb (*control*), an anti-β_1 mAb 4B4 (*β1*), an anti-α_9 mAb Y9A2 (*α9*), an anti-α_5 mAb P1D6 (*α5*), or an anti-α_4 mAb HP2/1 (*α4*). The data represent the means ± S.E. of at least two independent experiments in which triplicate determinations were made. *B*, photographs show morphology of G-361 cells adhering to FXIII in the presence of mAbs against various α subunits of integrin.

of β_1 paired with each of 12 distinct α subunits (α_1–α_{11} and α_v). Akimov *et al.* (32) have recently demonstrated that the integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ can mediate adhesion to tTG. In addition, Palmer *et al.* (9) reported that the α_9 subunit had high homology with α_4. Yokosaki *et al.* (14) demonstrated that the ligand binding sequence of tenascin-C, which is recognized by $\alpha_9\beta_1$-integrin, is homologous to the $\alpha_4\beta_1$ binding sequence in VCAM-1, and Taooka *et al.* (18) have shown that $\alpha_9\beta_1$ recognizes VCAM-1 as a ligand. $\alpha_{IIb}\beta_3$ is not generally expressed in cells other than platelets, but $\alpha_5\beta_1$ and $\alpha_9\beta_1$ are more widely expressed. We therefore explored the possibility that G-361 cells express $\alpha_9\beta_1$ and/or $\alpha_5\beta_1$ and that one or more of these integrins mediates adhesion to tTG. As depicted in Fig. 2, G-361 cells expressed $\alpha_9\beta_1$and a low level of α_5 but minimal α_4. In contrast, MOLT-3 cells, which adhere to tTG through $\alpha_4\beta_1$ (31), expressed a high level of α_4 but not $\alpha_9\beta_1$,

Adhesion of G-361 Cells to tTG Is Mediated by $\alpha_9\beta_1$- and $\alpha_5\beta_1$-Integrins—To determine whether $\alpha_9\beta_1$ is involved in adhesion of G-361 cells to tTG, we investigated the effect of anti-$\alpha_9\beta_1$ blocking mAb, Y9A2, on cell adhesion to tTG. TNfnIII3 peptide conjugated with BSA (TNfnIII3/BSA) was used as a positive control ligand for $\alpha_9\beta_1$ (14). As shown in Fig. 3*A*, adhesion to the conjugate was completely inhibited by this mAb, as was expected. Although adhesion of G-361 cells to tTG was inhibited only modestly by the anti-α_9-integrin mAb, the antibody dramatically inhibited cell spreading (Fig. 3*B*). As expected, anti-α4 mAb had no effect on either adhesion or spreading of these cells. These findings suggested that G-361 cell adhesion to tTG is at least partly mediated by $\alpha_9\beta_1$. However, because anti-$\alpha_9\beta_1$ only partially inhibited adhesion,

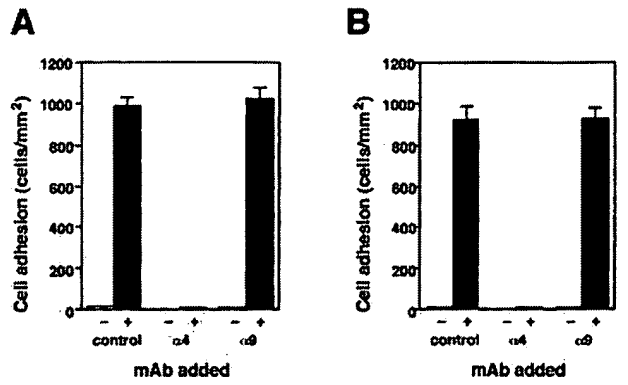

FIG. 6. **$\alpha_4\beta_1$-integrin-dependent adhesion of MOLT-3 cells to FXIII.** Adhesion of MOLT-3 cells to CS-1/BSA (*A*) or FXIII (*B*) was determined in the presence of following mAbs: *control*, without mAb; *α4*, an anti-α_4 mAb HP2/1; *α9*, an anti-α_9 mAb Y9A2. Assay was carried out in the absence (*striped columns*, −) or presence (*black columns*, +) of $MnCl_2$ and TS2/16 for stimulating integrins. The data represent the means ± S.E. of one of the representative experiments in which triplicate determinations were made.

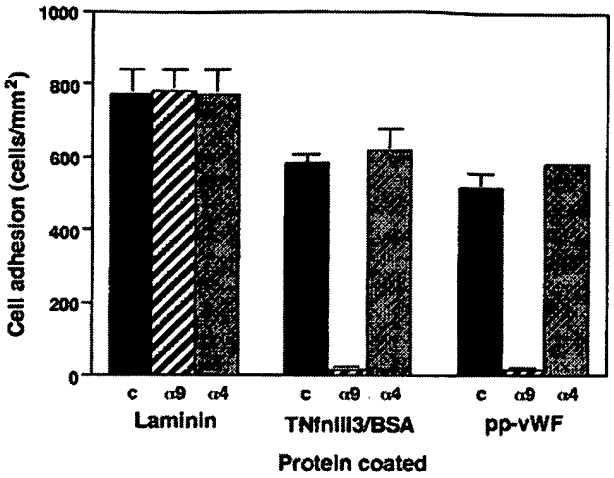

FIG. 7. **Adhesion of G-361 cells to pp-vWF is mediated by $\alpha_9\beta_1$-integrin.** Adhesion of G-361 cells to laminin, TNfnIII3/BSA, or pp-vWF was determined in the presence of no mAb (*black columns*, *c*), an anti-α_9 mAb Y9A2 (*striped columns*, *α9*), or an anti-α_4 mAb HP2/1 (*gray columns*, *α4*). The data represent the means ± S.E. of at least two independent experiments in which triplicate determinations were made.

whereas anti-β_1 antibody completely inhibited adhesion, our results suggest a role for other β_1-containing integrins (*e.g.* $\alpha_5\beta_1$) in this process. To determine which other integrins were involved, we examined the combined effects of anti-$\alpha_9\beta_1$ and other anti-integrin antibodies and peptides in cell adhesion to tTG. As depicted in Fig. 4, anti-α_5 mAb or RGD peptide dramatically augmented the inhibitory effect of the anti-$\alpha_9\beta_1$ mAb on adhesion of G-361 cells to tTG, whereas antibodies against α_2, α_3, α_4, or α_6 were without effect. Taken together, these results indicate that both $\alpha_9\beta_1$ and $\alpha_5\beta_1$ mediate adhesion of G-361 cells to tTG. These results, together with our previous report that $\alpha_4\beta_1$ is a receptor for tTG on MOLT-3 cells (31), suggest that tTG is an additional shared ligand for $\alpha_9\beta_1$ and $\alpha_4\beta_1$.

Adhesion of G-361 Cells to FXIII Is Mediated by $\alpha_9\beta_1$-Integrin—In a previous study (33), we reported that the active form of FXIII promoted cell adhesion of G-361 cells. We neither analyzed whether the activation of FXIII was necessary for the adhesion of G-361 cells nor determined the kind of integrins involved. In the present study, we show that the nonactivated form of FXIII also promotes G-361 cell adhesion. Both tTG and FXIII belong to the transglutaminase family and are highly homologous to each other. However, the recent study by Akimov *et al.* (32) reported that FXIII, in contrast to tTG, did not interact with $\alpha_5\beta_1$ (or any other integrins expressed on WI-38 human fibroblasts or human erythroleukemia cells, HEL). We speculated that the adhesion of G-361 cells to FXIII might be mediated by $\alpha_9\beta_1$, because this integrin is not expressed on WI-38 cells or HEL.[2] To confirm this, we investigated the effect of anti-$\alpha_9\beta_1$ mAb on adhesion of G-361 cells to FXIII. As shown in Fig. 5*A*, adhesion of G-361 cells to FXIII was almost completely inhibited either by mAb against β_1 or $\alpha_9\beta_1$, and the few cells that did adhere did not spread (Fig. 5*B*). The addition of mAbs against α_5- and α_4-integrins did not have any effect on adhesion or spreading. This result strongly indicates that the integrin, $\alpha_9\beta_1$, is the receptor for FXIII on G-361 cells.

Adhesion of MOLT-3 Human Lymphocyte-like Leukemia Cells to FXIII Is Mediated by $\alpha_4\beta_1$-Integrin and Not by $\alpha_9\beta_1$-Integrin—We have previously reported that MOLT-3 human lymphocyte-like leukemia cells did not adhere to FXIII (33). But at that time, we made no effort to activate integrins during

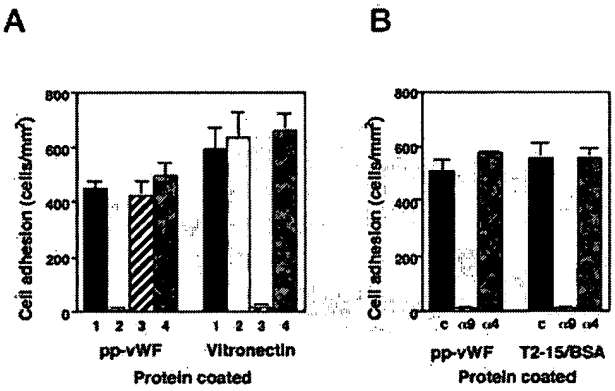

FIG. 8. **Identification of the essential adhesion site of pp-vWF recognized by $\alpha_9\beta_1$-integrin.** *A*, adhesion of G-361 cells to pp-vWF or vitronectin was determined in the absence (*black columns*, *1*) or presence of T2–15 peptide (*white columns*, *2*), RGD peptide (*striped columns*, *3*), or RGE peptide (*gray columns*, *4*). *B*, adhesion of G-361 cells to pp-vWF or T2–15/BSA was determined in the presence of no mAb (*black columns*, *c*), an anti-α_9 mAb Y9A2 (*striped columns*, *α9*), or an anti-α_4 mAb HP2/1 (*gray columns*, *α4*). The data are the means ± S.E. of one of the representative experiments in which triplicate determinations were made. *T2–15/BSA*, T2–15 peptide-BSA conjugate.

the cell adhesion assays. Masumoto and Hemler (34) described that $\alpha_4\beta_1$-mediated adhesion of leukocyte cell lines required activation of the integrin by divalent cations, stimulatory mAbs, or both. In fact, we previously showed that MOLT-3 cells adhered to tTG only when integrins were activated (31). We therefore reexamined adhesion of MOLT-3 cells to FXIII under conditions where integrins would be activated. As shown in Fig. 6*A*, integrin activation was needed for MOLT-3 cells to adhere to CS-1 peptide conjugated with BSA (CS-1/BSA). This adhesion was inhibited by anti-α_4-integrin mAb, as expected. As shown in Fig. 6*B*, MOLT-3 cell adhesion to FXIII also required activation, and this adhesion was completely inhibited by the mAb against α_4. These results indicate that MOLT-3 cells adhere to FXIII through $\alpha_4\beta_1$. Taken together, these results indicate that FXIII, as well as tTG, serves as a ligand for both $\alpha_9\beta_1$- and $\alpha_4\beta_1$-integrins.

Adhesion of G-361 Cells to pp-vWF Is Also Mediated by $\alpha_9\beta_1$—Previously, we reported that pp-vWF also mediated cell

[2] H. Takahashi, T. Isobe, S. Horibe, J. Takagi, Y. Yokosaki, D. Sheppard, and Y. Saito, unpublished observations.

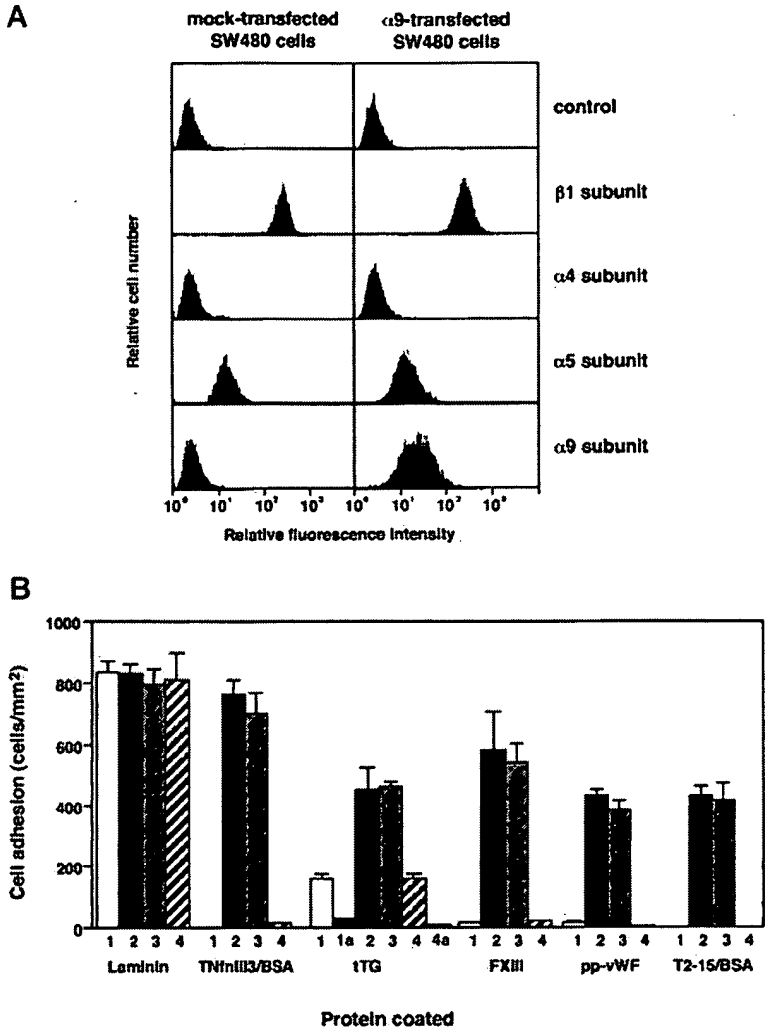

FIG. 9. **Adhesion of α_9-transfected SW480 cells to tTG, FXIII, and pp-vWF.** *A*, flow cytometry of integrins expressed on mock transfected and α_9-transfected SW480 cells. Cells were reacted with no IgG (*control*), an anti-human β_1 mAb TS2/16 (*β1 subunit*), an anti-human α_4 mAb HP2/1 (*α4 subunit*), anti-human α_5 mAb P1D6 (*α5 subunit*), or an anti-human α_9 mAb Y9A2 (*α9 subunit*). *B*, mock transfected SW480 cells (*white columns, 1*) or α_9-transfected SW480 cells were subjected to adhesion assays using various ligands. Adhesion of α_9-transfected SW480 cells to these ligands was assessed in the presence of no mAb (*black columns, 2*), an anti-α_4 mAb HP2/1 (*gray columns, 3*), or an anti-α_9 mAb Y9A2 (*hatched columns, 4*). In the case of tTG, mock transfected cells were also treated with the anti-α_5 mAb (*column 1a*) and α_9-transfected cells were also treated with anti-α_9 and anti-α_5 mAbs simultaneously (*column 4a*). The data represent the means ± S.E. of one of the representative experiments in which triplicate determinations were made.

adhesion through $\alpha_4\beta_1$ (24). We also reported that G-361 cells adhered to pp-vWF (30) and that this adhesion was not inhibited at all by an anti-α_4 mAb, but we were unable to define the responsible integrin(s). By analogy with tTG and FXIII, we have suspected that this adhesion might be mediated by $\alpha_9\beta_1$. As shown in Fig. 7, adhesion of G-361 cells to pp-vWF was completely inhibited by anti-$\alpha_9\beta_1$ mAb. As expected, adhesion to TNfnIII3/BSA was also completely inhibited by this mAb. Anti-$\alpha_9\beta_1$, however, did not inhibit adhesion of the cells to laminin, which is mainly mediated by $\alpha_6\beta_1$-integrin. This result indicates that the integrin $\alpha_9\beta_1$ is the receptor G-361 cells use for adhesion to pp-vWF. Thus, pp-vWF is also a ligand for both $\alpha_9\beta_1$- and $\alpha_4\beta_1$-integrins.

$\alpha_9\beta_1$- and $\alpha_4\beta_1$-Integrins Share the Same Cell Adhesion Site in pp-vWF—In a previous study (24), we demonstrated by experiments using synthetic peptides that an amino acid sequence within pp-vWF, DCQDHSFSIVIETVQ (designated as T2–15) was responsible for $\alpha_4\beta_1$-mediated adhesion of MOLT-3 cells to this protein. To determine whether $\alpha_4\beta_1$ and $\alpha_9\beta_1$ might share the same recognition sequence in pp-vWF, we performed cell adhesion assays in the presence of T2–15 or the irrelevant peptide, GRGDSP. As shown in Fig. 8*A*, adhesion of G-361 cells to pp-vWF was completely inhibited by the addition of T2–15 peptide, but the RGD peptide had no effect. On the other hand, adhesion to vitronectin, which is known to be mediated by RGD-dependent integrins, was inhibited by the RGD peptide, but not by T2–15 peptide. Furthermore, as depicted in Fig. 8*B*, G-361 cells adhered to T2–15 peptide conjugated with BSA (T2–15/BSA), and this adhesion was also completely inhibited by anti-$\alpha_9\beta_1$ mAb. This complete inhibition was also observed when cells adhered to intact pp-vWF. These results strongly suggest that $\alpha_9\beta_1$ and $\alpha_4\beta_1$-integrins share the same cell adhesion sequence within pp-vWF.

α_9 Subunit-transfected SW480 Human Colon Cancer Cells Adhere to tTG, FXIII, and pp-vWF—Using a single cell system, G-361 cells, we have defined tTG, FXIII, and pp-vWF as ligands for the integrin, $\alpha_9\beta_1$. To determine whether other cells could also use this integrin for adhesion to each of these ligands, we employed SW480 colon carcinoma cells that were stably transfected with either an α9-expression plasmid or empty vector (α_9-transfected SW480 cells and mock transfected SW480 cells, respectively). We determined by flow cytometry that only α_9-transfected SW480 cells expressed $\alpha_9\beta_1$, that neither SW480 cell line expressed α_4, and that both expressed similar levels of α_5 and β_1 (Fig. 9*A*). As shown in Fig. 9*B*, only α_9-transfected SW480 cells adhered to TNfnIII3/BSA, FXIII, pp-vWF, and T2–15/BSA. In each case, adhesion was inhibited almost completely by anti-$\alpha_9\beta_1$ mAb but not by anti-α_4 mAb. Mock transfected SW480 cells and α_9-transfected SW480 cells adhered equally well to laminin, indicating that all these cells were equally viable and that heterologous expression of α_9 did not nonspecifically augment cell adhesion. In the case of tTG, α_9-transfected SW480 cells adhered to the ligand, but mock transfected SW480 cells also adhered. The inhibitory effect of

TABLE I
Various adhesion ligands that are recognized by $\alpha_9\beta_1$ and/or $\alpha_4\beta_1$ integrin

Adhesion to various ligands is illustrated according to the results obtained by ourselves and other investigators. ○, recognition of the integrin; ×, no recognition of the integrin; ?, no determination. The proposed essential cell adhesion sequences are shown in parentheses.

	$\alpha_9\beta_1$	$\alpha_4\beta_1$
Fibronectin	×	○ (ILDV)
VCAM-1	○	○ (IDSP)
Tenascin-C	○ (AEIDGIEL)	?
Ostcopontin	○ (SVVYGLR)	○
pp-vWF	○ (DCQDHSFSIVIETVQ)	○
tTG	○	○
FXIII	○	○

the anti-α_9-integrin mAb on adhesion of α_9-transfected SW480 cells to tTG was not complete. In fact, the extent of the remaining adhesion activity was similar to that observed with mock transfected SW480 cells. Complete inhibition of adhesion of α_9-transfected SW480 cells to tTG was obtained by the simultaneous addition of anti-α_9- and anti-α_5-integrin mAb. The mock transfected SW480 cells did not adhere to tTG in the presence of the anti-α_5-integrin mAb. The most plausible explanation would be that $\alpha_5\beta_1$-integrin is also involved in cell adhesion to tTG, as was depicted in Figs. 3 and 4 using G-361 cells. These results indicate unambiguously that tTG, FXIII, and pp-vWF are adhesive ligands recognized by both $\alpha_9\beta_1$- and $\alpha_4\beta_1$-integrins, and that T2–15 within pp-vWF is the cell adhesion site that is recognized by $\alpha_9\beta_1$ and $\alpha_4\beta_1$.

DISCUSSION

Previous reports identified three ligands for the integrin $\alpha_9\beta_1$, VCAM-1 (18), tenascin-C (13, 14), and osteopontin (15, 16). In this report, we describe three additional ligands, tTG, FXIII, and pp-vWF. Among the previously identified $\alpha_9\beta_1$ ligands, VCAM-1 and osteopontin have both been reported to be recognized by both $\alpha_9\beta_1$ and the structurally related integrin $\alpha_4\beta_1$ (17, 35). In this report, we show that each of the three new $\alpha_9\beta_1$ ligands we identified is also a ligand for $\alpha_4\beta_1$.

Although it might seem reasonable to assume that $\alpha_9\beta_1$ and $\alpha_4\beta_1$ recognize the same adhesive sites in shared ligands, this is not necessarily the case. There are numerous examples of different integrins recognizing distinct sites in the same ligand. For example, at least five integrins can recognize the third fibronectin type III repeat in tenascin-C as a ligand, but four of them recognize the RGD site in the F-G loop, whereas $\alpha_9\beta_1$ clearly recognizes a different sequence, AEIDGIEL, in the B-C loop present on the same face of the protein (14). Similarly, both the integrins $\alpha_v\beta_3$ and $\alpha_9\beta_1$ recognize a thrombin-cleaved N-terminal fragment of osteopontin as a ligand (15), but $\alpha_v\beta_3$ binds to an RGD site, whereas $\alpha_9\beta_1$ binds to the adjacent sequence, SVVYGLR (16). In fact, prior to the present report, there was no definitive evidence that $\alpha_9\beta_1$ and $\alpha_4\beta_1$ could recognize the same site in a shared ligand. The evidence presented in this report that both integrins recognize the same linear peptide, T2–15, within pp-vWF is to our knowledge the first conclusive evidence that these integrins can recognize the same cell adhesion site within one molecule (Table I). Interestingly, the sequence of this peptide, DCQDHSFSIVIETVQ, bears little resemblance to the previously identified $\alpha_9\beta_1$-recognition sequences in tenascin-C and osteopontin. This observation provides additional evidence that $\alpha_9\beta_1$ can recognize a surprisingly broad array of adhesive ligands. Taken together,

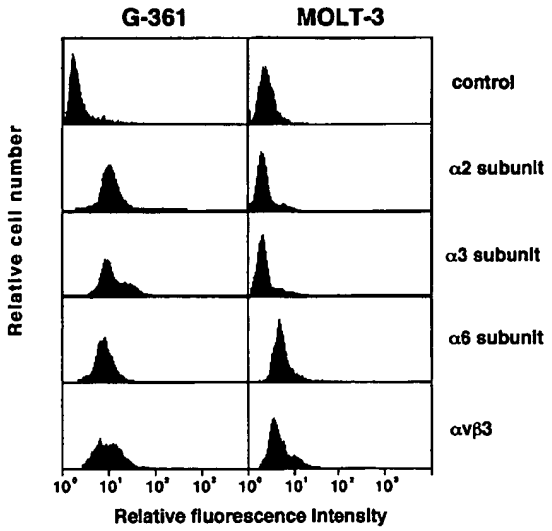

FIG. 10. **Flow cytometry of integrins expressed on G-361 cells and MOLT-3 cells.** Cells were reacted with no IgG (*control*), an anti-human α_2 mAb 6F1 (*α2 subunit*), an anti-human α_3 mAb P1B5 (*α3 subunit*), an anti-human and -mouse α_6 mAb GoH3 (*α6 subunit*), or anti-human $\alpha_v\beta_3$ polyclonal antibodies (*αvβ3*).

the accumulated evidence that $\alpha_9\beta_1$ and $\alpha_4\beta_1$ share five ligands and, at least in one instance, recognize the same linear peptide within a ligand confirms the utility of α subunit sequence comparisons for predicting ligand binding specificity of integrin heterodimers.

The interaction between tTG and integrins is likely to be biologically significant. tTG directly binds to a number of components of the extracellular matrix, including osteopontin and tenascin-C, where it plays an important role in matrix protein cross-linking. A recent report demonstrated that tTG bound to the integrins $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ within the secretory apparatus prior to appearance of the integrins on the cell surface, thereby suggesting a mechanism for localization of tTG to its extracellular targets (32). Furthermore, the same study showed that the interaction of integrins with tTG enhanced cell attachment and spreading on fibronectin. The findings in the present study greatly expand the complexity of these potential interactions and suggest that similar mechanisms could be involved in cellular interactions with additional sites in fibronectin (*e.g.* the CS-1 site) and with a broad array of extracellular matrix ligands. Recently, as noted above, Akimov and co-workers reported that tTG could interact with several members of the integrin family, including $\alpha_5\beta_1$ (as we also report in the current study), but also $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (32). Interestingly, the report by Akimov *et al.* demonstrated that the related transglutaminase family member, FXIII, was not a ligand for $\alpha_5\beta_1$, and thus suggested that the interactions between integrins and transglutaminases was specific for tTG. Although that report and this one both described interactions between $\alpha_5\beta_1$ and tTG, there were several substantial differences. For example, Akimov *et al.* found no effect of either GRGDSP peptide or anti-β_1 antibody on the interaction between tTG and integrins they describe, whereas in this report we found that GRGDSP completely eliminated the residual adhesion of G-361 cells to tTG once $\alpha_9\beta_1$-mediated adhesion was inhibited, and we also found complete inhibition with anti-β_1 antibody. Furthermore, we found no role for integrins other than $\alpha_4\beta_1$, $\alpha_5\beta_1$, or $\alpha_9\beta_1$ in the adhesion of MOLT-3 or G-361 cells to tTG. We have checked the expression of various integrin subunits other than those shown in Fig. 2. As is clearly depicted in Fig. 10, G-361 cells also expressed α_2, α_3, α_6, and $\alpha_v\beta_3$. MOLT-3 cells, how-

ever, did not express these integrins substantially. Results obtained with G-361 cells further substantiate the notion that these integrins are not involved in the adhesion of G-361 cells to tTG. There are several possible explanations for these differences. One obvious difference is that Akimov *et al.* were examining the effects of tTG on cell adhesion to the 42-kDa fragment of fibronectin, and in their case the tTG was supplied to integrins within the secretory apparatus of the same cell, whereas the present study examined adhesion to immobilized tTG. Under the conditions examined by Akimov *et al.*, it is conceivable that blocking antibodies and GRGDSP peptide would not be able to displace already bound tTG, whereas these reagents were perfectly capable of binding to unligated integrin under the conditions used in the present study, thereby blocking subsequent interactions with tTG. Such an explanation is plausible because it is often more difficult to detach already adherent cells with integrin-blocking reagents than to block the attachment of suspended cells. Alternatively, the WI-38 cells and HEL cells used by Akimov *et al.* and the MOLT-3 cells and G-361 cells used in the present study could interact with tTG through different mechanisms.

The results of the present study also clearly demonstrate that although $\alpha_5\beta_1$ is not a receptor for inactive FXIII, both $\alpha_4\beta_1$ and $\alpha_9\beta_1$ are. These findings appear to differ from our previous report suggesting that $\alpha_5\beta_1$ did contribute to adhesion of TIG-1 cells to FXIII. However, the adhesion observed in those experiments required that the FXIII be activated. The results of the present study confirm that $\alpha_5\beta_1$ on G-361 cells can also mediate adhesion to activated FXIII (data not shown). We are uncertain whether the requirement for activation in this case is due to a conformational change in FXIII upon activation that unmasks an $\alpha_5\beta_1$ binding site or to a requirement for enzymatic activity to induce $\alpha_5\beta_1$-mediated adhesion

The interaction of $\alpha_4\beta_1$ and $\alpha_9\beta_1$ with FXIII could be biologically significant. Both $\alpha_4\beta_1$ and $\alpha_9\beta_1$ are highly expressed on specific populations of leukocytes, where they play a prominent role in transendothelial leukocyte migration. As a member of the coagulation cascade, FXIII is likely to be enriched at sites of vascular injury and inflammation, where its interaction with $\alpha_4\beta_1$ and $\alpha_9\beta_1$ could promote leukocyte extravasation.

We speculated previously about the function of pp-vWF as an emergency tag for targeting of $\alpha_4\beta_1$-expressing leukocytes, together with VCAM-1, to sites of inflammation and injury (24). Such a mechanism would be expected to be most relevant to lymphocytes, monocytes, and eosinophils, all of which express high levels of $\alpha_4\beta_1$ (36). The recent report by Taooka *et al.* (18) that human neutrophils express $\alpha_9\beta_1$ and utilize this integrin for migration on VCAM-1 and through activated endothelial monolayers extends this possible function to all populations of circulating leukocytes.

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